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Renal extracellular vesicles: from physiology to clinical application

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Abstract

Urinary extracellular vesicles (uEVs) are released from all regions of the kidney's nephron and from other cells that line the urinary tract. Extracellular vesicles retain proteomic and transcriptomic markers specific to their cell of origin and so represent a potential reservoir for kidney disease biomarker discovery. Exosomes, a subtype of uEVs, are distinguished from other vesicles by features related to their biogenesis within cells: mature multi-vesicular bodies fuse with the cellular membrane to liberate exosomes into the extracellular space. uEVs represent a novel cell signalling mechanism because they can be shuttled to a recipient cell and, through a number of proposed mechanisms, affect the recipient cell's proteome and function. Here we review the current evidence for uEV signalling along the nephron, their role in health and disease of the kidney, and potential for clinical translation as biomarkers and therapeutics.

Introduction

The kidney is the one of the most important regulators of the body's physiological state, manipulating filtration and reabsorption of solutes in order to maintain an optimal environment for health. The kidney is vulnerable to a plethora of injury modalities. High oxygen demand and low tissue oxygen tensions in the renal parenchyma sensitise tubular cells to hypoxia and can lead to acute and chronic kidney injury, disease processes which are associated with substantial morbidity and mortality. Furthermore, tubular cells are vulnerable to the toxic effects of drugs. Increasing intra-tubular drug concentrations as the filtrate moves along the nephron combined with reuptake mechanisms for solutes result in potentially toxic intracellular drug concentrations. As a result of the high morbidity associated with renal disease, and the limiting role of nephrotoxicity in translation of drug development to clinical practice, improving our understanding of the underlying molecular signalling would be of value to prevent toxicity and treat kidney injury (Lee *et al.* 2014).

Urine, the excreted filtrate of the kidney, is unique in providing a non-invasive snapshot of the kidney's function. This filtrate is composed of ions, inorganic and organic compounds (including proteins, hormones and metabolites) suspended in water. Investigation of the protein fraction of urine has demonstrated the presence of integral membrane proteins within small extracellular vesicles (EVs) (Wen *et al.* 1999). EVs are parcels of proteomic and transcriptomic information. Proteins, pre-microRNA, mature microRNA, retrotransposon RNA transcripts, single-stranded DNA (ssDNA), double-stranded dsDNA and mitochondrial DNA have all been identified within EVs (*abstract figure*) (Guescini *et al.* 2010, Balaj *et al.* 2011, Thakur *et al.* 2014). Rigorous characterisation of the urinary EV's (uEVs) protein and RNA content has been conducted, identifying molecules unique to all regions of the nephron and identifying products of genes associated with multiple disease processes (Pisitkun *et al.* 2004, Miranda *et al.* 2010). Several subtypes of vesicles have been identified in urine, including exosomes and microvesicles, the characteristics of which are described below (Pisitkun *et al.* 2004, Rood *et al.* 2010).

Composition and biogenesis of EVs

EVs encompass a vast heterogeneous and dynamic population of membrane bound vesicles; their content and membrane composition is not only dependent upon their cellular source but is also sensitive to cellular stress and environmental changes. Historically, naming of EVs was dependant upon their cell of origin e.g. cardiosomes (cardiomyocyte origin), prosatosomes (seminal fluid). Current nomenclature, however, largely distinguishes vesicles by their biogenesis. Criteria for EV classification have been proposed based on their origin, function or biogenesis; yet there is still no consensus about their nomenclature. Subpopulation categories range from 3-6 in number in most reviews and have been extensively discussed elsewhere (Thery *et al.* 2009, van der Pol *et al.* 2012). These categories are often difficult to use in practice, which results in confusing overlapping nomenclature. For the purposes of this review, we will consider three distinct subtypes with characteristics related to their origin, size and identifying markers:

Exosomes

Exosomes are derived from the endosomal pathway. Transmembrane proteins are trafficked to early endosomes by endocytosis. These early endosomes undergo sorting to late endosomes, which in turn become multivesicular bodies (MVBs). Formation of MVBs is directed by the recruitment of proteins and budding of intraluminal vesicles (ILVs), via the endosomal sorting complex required for transport (ESCRT). ILVs are released as exosomes upon fusion of MVBs with the plasma membrane (*figure 1*). Alternatively, MVBs can fuse with lysosomes, resulting in degradation of their contents.

Intriguingly, the relative abundance of cellular RNA and protein does not closely correlate with that of the exosome, suggesting a selective process of entry into vesicles (Valadi *et al.* 2007). This cargo sorting may allow the cell to generate exosomes of precisely defined biochemical composition. ESCRT, tetraspanins and lipid-dependent mechanisms have all been implicated in the selective loading of proteins (Villarroya-Beltri *et al.* 2014). A specific pattern of protein post-translational modifications (PTMs) has been noted in exosomes and a potential role in exosome sorting has been suggested (Moreno-Gonzalo *et al.* 2014). The most commonly described PTM, ubiquitination, involves the addition of ubiquitin to a target protein (*figure 1*). In MVBs, a ubiquitinated protein is sorted into ILVs by the ESCRT and the downstream fate of the MVB is dependent upon the type of ubiquitin modification on a specific substrate. Ubiquitin may be cleaved from its cargo protein during incorporation to the ILV but a recent *in vivo* study reported that 15% of proteins in uEVs are ubiquitinated, suggesting a significant proportion are sequestered into ILVs without deubiquitination (Agromayor *et al.* 2006, Huebner *et al.* 2016). Others PTMs that have been described include phosphorylation and glycosylation (Moreno-Gonzalo *et al.* 2014).

The sorting of RNA into exosomes is less well understood; specific small ubiquitin-related modifier (SUMO) proteins are hypothesized to interact with cis-acting elements within RNA,

such as hnRNPA2B1, to selectively load vesicles (Villarroya-Beltri *et al.* 2013). MiRNA sorting to exosomes may be modulated by the dynamic transcriptomic changes seen in cell activation, differentially engaging miRNAs at P bodies or MVBs (Squadrito *et al.* 2014).

As a result of the above processes, exosomes represent a parcel of protein, DNA and various RNA species (Sheldon *et al.* 2010, Mittelbrunn *et al.* 2011). The majority of RNA species isolated from exosomes are small RNAs including microRNAs (miRNAs) (Cheng *et al.* 2013). Exosomes are identified by their lipid bilayer membrane, 30–120 nm size and density of 1.15–1.19 g/ml in continuous sucrose gradient (Thery *et al.* 2006). Their often cited cup-shaped morphology is likely artificial, attributed to collapse during drying (Raposo *et al.* 2013). An exosome's lipid bilayer outwardly displays the apical surface of the membrane from which the vesicle was formed, therefore displaying the same extracellular surface markers as the cell of origin (*figure 1*) (Thery *et al.* 2009). This membrane orientation has led to the postulation of inward budding, from the limiting membrane of endosomes, as the mechanism of exosome biogenesis. Exosomal protein markers mainly relate to intra-cellular vesicle trafficking and exosome biogenesis i.e. ESCRT components, tetraspanins and flotillin. Although there are signature protein profiles for exosomes from defined tissue, there is no single unifying marker. Specific to the urinary exosome population, CD24 has been postulated as a suitable biomarker (Keller *et al.* 2007, Oosthuyzen *et al.* 2013).

Microvesicles

Microvesicles are shed directly from the plasma membrane via detachment of small cytoplasmic protrusions (Yanez-Mo *et al.* 2015) in response to cell stress. This process is dependent on calcium influx, calpain, and cytoskeleton reorganization (*figure 1*) (Cocucci *et al.* 2009). As a result of this outward budding microvesicles contain a small volume of cytoplasm and are enriched with membrane markers from their cell of origin including proteins associated with membrane lipid rafts (Del Conde *et al.* 2005). Microvesicles are characteristically 50 - 1000nm in size and specific markers proposed for their identification are ADP-ribosylation factor 6 (ARF6; implicated in endocytosis of protein) and vesicle-associated membrane protein 3 (VAMP3) (Muralidharan-Chari *et al.* 2009).

Consideration can also be given to demonstrating the absence of non-EV protein markers when screening exosome and microvesicle populations. For example transferrin receptors are enriched in the exosomes but are absent in the microvesicle population from the same tissue (Muralidharan-Chari *et al.* 2010).

Apoptotic bodies

Apoptotic bodies are blebs containing cytoplasm and densely packed organelles. They are extensively liberated from the plasma membrane in the later stages of apoptosis. Although apoptotic bodies are generally considered to be larger in size than other vesicles (500-4000nm),

a smaller subpopulation has been proposed, 50-500nm (Thery *et al.* 2009, Akers *et al.* 2013). The translocation of phosphatidylserine onto the outer cell membrane during apoptosis has been previously described and it is unsurprising that apoptotic bodies are enriched for this phospholipid. Binding of annexin V to phosphatidylserines therefore can be used as a protein marker for apoptotic bodies. Phosphatidylserines are also exposed by microvesicles and, to a lesser extent, exosomes. Increased binding sites for thrombospondin and C3b allow these proteins to be used as markers (van Engeland *et al.* 1998). Given the circumscribed pathophysiological role of apoptotic bodies and their efficient local clearance by phagocytosis *in vivo*, apoptotic bodies will not be discussed in further detail in this review.

Pisitkun *et al.* first described the presence of uEVs, identifying these as exosomes due to their small size and biogenesis, and describing the proteome of these vesicles (Pisitkun *et al.* 2004). Since this seminal work was published, the presence of different vesicle subtypes has also been described (Rood *et al.* 2010). Over recent years, a wealth of studies have gone on to describe both the proteome and transcriptome of the uEV population (Gonzales *et al.* 2009, Miranda *et al.* 2010), culminating in the development of public access online databases (<http://www.exocarta.org>, <https://hpcwebapps.cit.nih.gov/ESBL/Database/Exosome/>)

Isolation of a pure vesicle sub-population is notoriously difficult, in part because of the size overlap between the vesicle sub-populations, and other vesicles are often co-purified along with the specific type of interest. Due to inconsistency in EV isolation protocols and often-incomplete vesicle characterization, there is an inherent difficulty in differentiating between the subpopulations in published work. For clarity, in this review vesicle like structures that are not rigorously defined will be referred to as EVs or uEVs unless clearly stated.

uEV concentration and quantification

Technical standardisation of sample processing is an area of controversy in EV research, although the influence of varying practice on downstream outputs remains unclear. Variability exists in storage, handling and characterisation of uEVs between published works; fortunately guidance is now available through position papers of best practice and consensus statements on EV isolation and minimal experimentation requirements for EV definition (Witwer *et al.* 2013, Lotvall *et al.* 2014).

Storage and handling of uEVs

Fresh urine samples are preferable for isolation of uEVs, although for practicality frozen samples may need to be used with like-for-like being compared (i.e. fresh-to-fresh, frozen-to-frozen). EVs are relatively insensitive to freeze/thaw cycles and may even resist bursting in a hypotonic environment (Witwer *et al.* 2013). Storage of urine at -80°C is appropriate and uEVs have been concentrated from samples after 7 months in storage (Zhou *et al.* 2006, Oosthuyzen

et al. 2013). Use of protease inhibitors to preserve samples has been described, but opinion remains inconsistent regarding their use (Zhou *et al.* 2006, Oosthuyzen *et al.* 2013). Calcium oxalate and amorphous calcium crystal precipitates can be present after thawing, macroscopically forming a cloudy sample. Vigorous vortexing can re-dissolve these salts (Saetun *et al.* 2009).

Tamm-Horsfall protein (THP; also known as uromodulin) is an abundant protein in urine and leads to uEV entrapment by polymerising and co-precipitating at low speed centrifugation, leading to a reduced yield of uEVs in the final ultracentrifugation pellet (Fernandez-Llama *et al.* 2010, Kosanovic *et al.* 2014). Removal of THP is therefore recommended either with DTT (dithiothreitol), which disrupts the zona pellucida disulphide bonds of THP, or CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic), a mild solubilising detergent (Fernandez-Llama *et al.* 2010, Musante *et al.* 2012, Witwer *et al.* 2013, Lotvall *et al.* 2014). Although the latter preserves protein conformation and enzymatic activity, it is significantly more time consuming to use.

Ultracentrifugation

Differential ultracentrifugation, with or without a size exclusion technique, remains the most accepted method of exosome isolation from biological fluids (*table1*) (Harding *et al.* 1983, Johnstone *et al.* 1984). Following cell depletion of a biological fluid by slow speed centrifugation, 10,000 to 20,000*g* is used to pellet larger EVs. Subsequently, smaller EVs, including exosomes, are pelleted from this supernatant using an ultracentrifuge (100,000 to 200,000*g*). The limitations of this technique have previously been well described in other reviews; ultracentrifugation introduces variability, has a low through put, and is heavily operator and rotor dependent (Cvjetkovic *et al.* 2014). Importantly, not all exosomes are recovered following ultracentrifugation; 40% remain in the supernatant although it is unknown if this subpopulation contains unique features of biological/clinical relevance (Musante *et al.* 2012). Size exclusion chromatography can be used in conjunction with ultracentrifugation to remove protein contaminants and is considered the gold standard for isolation of a highly purified population of morphologically intact exosomes (Rood *et al.* 2010, Boing *et al.* 2014).

Alternatively, filtration through a nano-membrane, using slow centrifugation or gravity, can further aid size exclusion and theoretically improve purity of the vesicle subpopulation. Limitations of this technique include a lower EV recovery, vesicle fragmentation and contamination with proteins, with further protein retention on the membrane (Cheruvanky *et al.* 2007, Witwer *et al.* 2013). Addition of a sucrose gradient can further improve purity and isolate subpopulations, but this process is lengthy and associated with a very low EV yield (<1% of the initial crude pellet) (Hogan *et al.* 2014). Interestingly, sucrose gradient ultracentrifugation has been used to elucidate 2 different subtypes of exosome population within melanoma cell supernatant. The populations were distinct in their proteome, transcriptome and effect on

recipient cell gene expression; raising the possibility of heterogeneous exosome population capable of exerting different effects on downstream cell physiology (Willms *et al.* 2016).

Other concentration techniques

A number of commercial platforms support rapid EV precipitation. These proprietary polymers pellet EVs, with the aid of a slow centrifugation step, and have been used for a wide variety of downstream applications. This approach is attractive due to the rapid aggregation, low user variability and relative simplicity (Schageman *et al.* 2013, Musante *et al.* 2014). However, caution must be exercised as these techniques also concentrate larger vesicles and protein aggregates, forming an impure pellet (Alvarez *et al.* 2012). Immunoaffinity precipitation has been used in a number of studies to concentrate purified subpopulations of EVs, utilising magnetic beads coated with antibody against proteins of interest (Kalra *et al.* 2013, Wang *et al.* 2014). Similar peptide-based isolation techniques have also been described (Ghosh *et al.* 2014). Although these techniques have the capability to achieve an adaptable, rapid platform for EV isolation, they do, inherently, introduce population bias due to targeting only vesicles that express a certain protein marker (Kowal *et al.* 2014).

Novel microfluidic devices utilise a variety of techniques; namely, immunoaffinity, sieving (through nanoporous membranes) and trapping of vesicle structures, to identify a highly purified EV population (Kanwar *et al.* 2014, Santana *et al.* 2014, Liga *et al.* 2015). Although in its infancy, this technique could be used in point-of-care rapid exosome isolation and has been comprehensively reviewed elsewhere (Liga *et al.* 2015). There remain a number of hurdles to employment of this technique, mainly low recovery of exosomes, sheer stress to structures, and requirement for prior sample preparation.

Normalisation

Total EV number can be ascertained by nanotracking analysis (NTA) without or with fluorophores to known protein targets, with the additional benefit of using unprocessed urine samples (Oosthuyzen *et al.* 2013). This methodology could be used to sample 2 populations (the subpopulations of interest and a control) or even dichromatic populations (Hogan *et al.* 2014). Alternatively, manipulation of pore size of a stretchable nanopore membrane, utilised in resistive pulse sensing, can allow for measurement size distribution and concentration but this approach is limited by the inability to provide phenotypic information (Liga *et al.* 2015). The primary issue with NTA and nanopore technologies include lengthy processing and inter-assay variability. Until recently conventional flow cytometry could only phenotype EVs down to ~500nm in size, restricting its use to the study of larger vesicle populations. The advent of newer instruments has raised the exciting possibility of discriminating between particles as small as 100 nm in diameter (Witwer *et al.* 2013). The impact of different refractive indices between biological, silica and polystyrene microparticles, and the resultant possibility of confounding results, remains debatable (Mullier *et al.* 2011, Witwer *et al.* 2013). This issue is

further complicated in the study of uEVs, as calcium-phosphate microprecipitates have been shown to overlap the EV population on the flow cytometry signal (Larson *et al.* 2013).

Given the limitations of describing a total uEV population, alternative normalisation methods across samples are needed to allow valid analysis of proteomic and transcriptomic changes. This has previously been described 'the holy grail' of uEV study, with a number of well described limitations (Salih *et al.* 2014). Theoretically, 2 broad approaches exist. Firstly, normalisation with a defined time period (time-normalisation) or secondly, by a housekeeping marker (protein-normalisation). Instinctively, the former approach would appear to be the optimal, but obtaining a timed urine collection can be difficult in clinical practice. Therefore, urinary creatinine is commonly used as a surrogate with the assumption that uEV concentration is correlated to urine concentration. Protein-normalization (normalizing to a target housekeeping protein) can also be used. Example proteins include: CD9, CD63, CD81, TSG-101, and ALIX (Street *et al.* 2011, Alvarez *et al.* 2012). This approach assumes the normalising protein urinary excretion does not change in different disease states and there is no biological/clinical relevance to a change in the number of vesicles within the sample. Despite the different underlying principles, there is no consensus regarding the optimal approach for normalisation.

Biomarker discovery in EVs

In both acute and chronic kidney disease current biomarkers, such as creatinine, focus on the recognition of established disease rather than early detection of imminent renal dysfunction. These investigations provide little information about the underlying pathophysiology and renal biopsy is considered the gold-standard investigation. Renal biopsy is an invasive technique with a number of adverse events such as infection and haemorrhage. Also biopsy cannot be performed serially (daily) and is prone to sampling error. There is hope that harnessing the proteomic and transcriptomic changes of uEVs in varying disease states will present a non-invasive alternative to biopsy. As discussed, uEVs are released into urine from all regions of the nephron and are readily identified by proteins specific to the cell of origin, potentially providing a non-invasive snapshot of the physiological state of nephron (Pisitkun *et al.* 2004, Alvarez *et al.* 2012). uEVs represent a remarkably stable, easily accessible, biomarker reservoir, which protects its cargo from the harsh extra vesicular environment. uEVs are promising biomarker candidates, with potential to predict disease, define mechanisms and prognosticate.

3 fundamental biomarker development stages have previously been described; biomarker *discovery*, *validation* of the markers predictive value within the population and *implementation* of a clinically approved assay (Granger *et al.* 2004, Pisitkun *et al.* 2012). In relation to uEVs, biomarker discovery in well-defined populations has identified a number of targets. *Table 1* summarises the candidate EV biomarkers to date, distinguishing biomarkers of interest by disease process. In models of kidney injury, the proteins in the exosomal fraction have been reported to change prior to elevation in the 'free' fraction in urine and, importantly, prior to traditional biochemical and histological diagnostic tests (Zhou *et al.* 2006, Alvarez *et al.* 2013). Lower EV recovery rates in patients with heavy proteinuria and the outlined difficulties in normalisation have led investigators to pursue qualitative targets i.e. the presence or absence of the target defines disease or health. Ultimately, quantitative measurements would be desirable, allowing the ability to track deterioration or improvement in the clinical condition. The validation phase of study, and the challenges to further progress, perhaps best reflect the current situation. Large population studies are required, outwith the population of interest, to identify both the positive and negative predictive value of the EV-based biomarker. Several studies have investigated the selectivity of uEVs but few have interrogated the specificity. The practicality and cost of the necessary large-scale clinical studies limits further progress, as sample processing and quantification are not currently translatable to hundreds or thousands of samples.

Function

Inter-cellular communication depends upon an EV's ability to influence a recipient cell, either by receptor-mediated interaction, endocytosis of the EV or fusion of the vesicle membrane to the plasma membrane. This latter signalling mechanism results in delivery of an EV's contents

directly into the cytoplasm; including transcription factors, miRNA, mature RNA and infective particles (Ratajczak *et al.* 2006, Valadi *et al.* 2007, Cocucci *et al.* 2009, Camussi *et al.* 2010).

Physiology

Along the nephron, uEV-mediated intercellular signaling has been postulated to explain why proximal tubule proteins are present in downstream nephron segments (van Balkom *et al.* 2011, Dear *et al.* 2013, Okada 2013), but this has not yet been conclusively demonstrated *in vivo* (van Balkom *et al.* 2011). uEVs are released into urine from all regions of the nephron and can be readily identified by transcriptomic and proteomic markers specific to the cell of origin (Pisitkun *et al.* 2004, Alvarez *et al.* 2012). Hypothetically, via uEV release and downstream reuptake, uEV contents could affect the function of a downstream recipient cell (Dimov *et al.* 2009). Notably, the exosomal fraction of aquaporin-2 (AQP2) increases in response to desmopressin and transfer of uEVs from desmopressin-treated cells to untreated cells results in an increase of functional AQP2 expression in the recipient cell (Street *et al.* 2011, Higashijima *et al.* 2013). Cortical collecting duct cells stimulated with vasopressin take up ECVs, *in vitro* and *in vivo*. This process can be manipulated to deliver miRNA to collecting duct cells resulting in downregulation of target transcripts (Oosthuyzen *et al.* 2016). This study demonstrated that uEV signaling is a physiologically regulated process, which can be manipulated to deliver miRNA. Interaction of uEVs with recipient cells may involve specific interaction with primary cilia, as reported with polycystic disease-positive vesicles using transmission electron microscopy images (Woollard *et al.* 2007, Hogan *et al.* 2009). This observation is supported by data from a biliary model that demonstrate exosome signaling affects ERK signaling, miRNA expression and cell proliferation, with abolishment of this signal following removal of cilia (Masyuk *et al.* 2010). This work, in conjunction with the observation of multiple protein products of genes known to be responsible for renal and systemic diseases in normal urine, raises interesting questions about the role of EV signalling in health and disease (Pisitkun *et al.* 2004).

Pathophysiology

uEV signalling has been implicated in the pathogenesis of acute kidney injury (AKI): exosomes from injured tubular cells transfer TGF- β 1 mRNA into fibroblasts, resulting in cell activation (Borges *et al.* 2013). *In vitro*, vesicles appear to be important in mediating vascular smooth muscle cell calcification, a potential mechanism for accelerated vascular calcification in end stage renal disease (Reynolds *et al.* 2004). Interestingly, the paracrine effect of liver stem cells has been shown to aid regeneration of renal tubular injury via release of EVs, highlighting the possible beneficial role of exosome signalling in systemic illness (Herrera Sanchez *et al.* 2014).

Mesenchymal stem cells (MSC) accelerate recovery and repair tissue following AKI; an effect demonstrated using diverse injury modalities (Asanuma *et al.* 2010, Reinders *et al.* 2010, Togel *et al.* 2012, Wise *et al.* 2012, Fleig *et al.* 2014). Whether this reflects direct cell engraftment and differentiation, or is mediated through release of paracrine factors is unclear. However, the

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observation that the MSC supernatant conferred a beneficial effect, equal to that of MSCs themselves, has opened the field to EV research (Bi *et al.* 2007). Seminal work by Bruno *et al.* supported the claim that this positive result was largely due to EV transfer, likely related to RNA interference (Gatti *et al.* 2011, Biancone *et al.* 2012, Bruno *et al.* 2012, Cantaluppi *et al.* 2012, Zhou *et al.* 2013). Recent work suggests EV miRNA has a significant role in this MSC effect on kidney cell injury (Collino *et al.* 2015). Notably, miRNAs associated with EV endothelial progenitor cells reduced apoptosis and promoted cell regeneration in ischemia reperfusion injury (Bitzer *et al.* 2012, Cantaluppi *et al.* 2012, Chen *et al.* 2014). This possible role of EV signalling in the delivery of functional miRNA and pathogenesis of AKI highlights their potential as a therapeutic intervention. Abnormal levels of miRNA could be one of the mechanisms explaining dysregulated protein expression during kidney disease progression and interference with this process represents a potential therapeutic target (Ho *et al.* 2012).

uEVs, in addition, may also act as antibacterial immune effectors, mediating the host response to urinary tract infection by inhibiting growth of pathogenic and commensal *Escherichia coli* and inducing bacterial lysis (Hiemstra *et al.* 2014). This highlights the multimodality capacity of EVs in therapeutics, specifically their potential as novel antibiotics for urinary tract infections, a common illness affecting 150 million patients annually (Flores-Mireles *et al.* 2015). Furthermore, renal brush border-derived exosomes can induce calcium oxalate crystallization in nephrolithiasis and may have a role in renal stone disease although this mechanism is yet to be demonstrated *in vivo* (Khan 2004).

Potential as therapeutics

The contribution of EV signalling in health and disease highlights their potential as attractive therapeutic targets and there are a number of on-going phase I and II clinical trials harnessing EV-based therapeutics. Although we remain in the early phase of such studies, theoretical clinical utility could be mediated by interfering with EV biogenesis or the manipulation of EVs as therapeutics vectors.

Vectors for drug delivery

EVs are candidate drug delivery systems; they are stable vehicles with a wide biodistribution. They can be selectively loaded and can deliver functional RNA into cells. The integrity of RNA isolated from vesicles is similar to that of tissue and far higher than RNA in whole urine, as the membrane protects the RNA cargo from RNase degradation (Miranda *et al.* 2010, Cheng *et al.* 2013). Interestingly, EVs have natural targeting capacity, presumably by receptor-ligand binding (Sun *et al.* 2010, Zhuang *et al.* 2011, Tian *et al.* 2014). Recent work, conducted by Hoshino *et al.* (Hoshino *et al.* 2015) demonstrated tissue-specific uptake of EVs is mediated by distinct integrins via their interaction with the extracellular matrix of the target tissue. Manipulation of this mechanism, through therapeutic targeting of these integrins, reduced EV uptake and impeded metastatic spread of cancer. This ability to predict the metastatic course of cancer raises the exciting possibility of prediction and redirection of tumour progression. A similar mechanism could also be responsible for EV signalling along the kidney and the observation of proximal tubular specific proteins in distal segments raises the possibility this

process occurring *in vivo*. EV signal manipulation *in vivo*, to target exogenous vesicles to the tissue of interest through delivery of miRNA and siRNA, has already been demonstrated, ultimately affecting downstream gene expression (Alvarez-Erviti *et al.* 2011, Bryniarski *et al.* 2013). Furthermore, bioengineered nanoparticles can serve as exosome mimics, recreating these functions and delivering targeted chemotherapeutics (Jang *et al.* 2013).

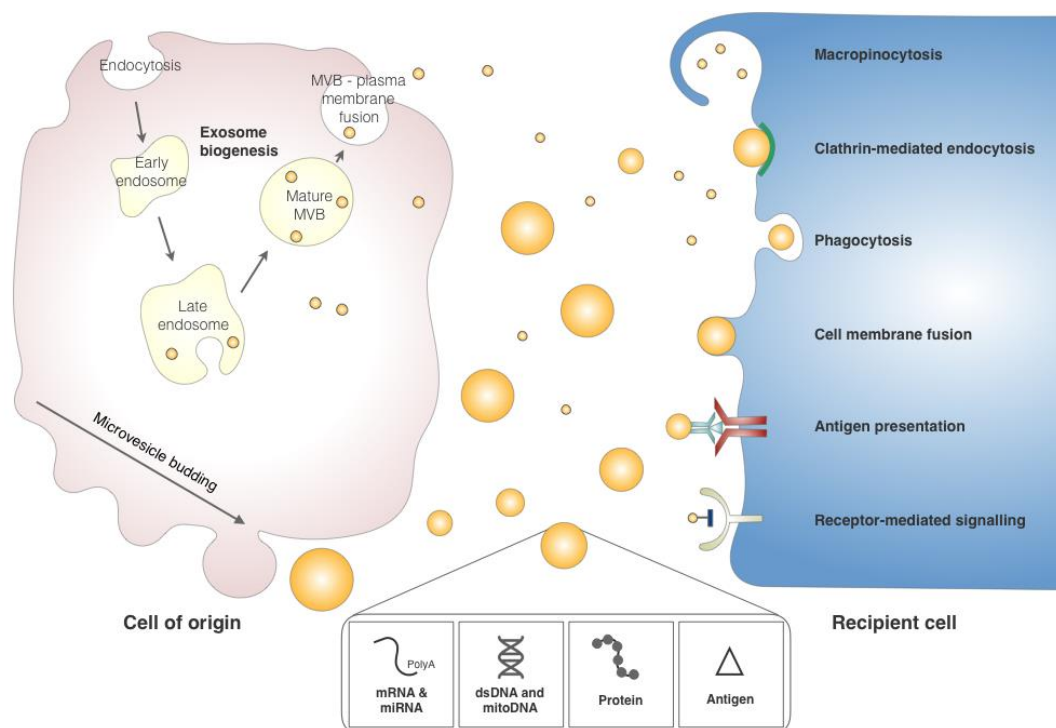
Inhibiting EV biogenesis and uptake

The circulating concentration of exosomes has been correlated to cancer progression and overall survival, which suggests that reducing exosome numbers may be a potential therapeutic approach. Proof-of-concept using amiloride (an antihypertensive agent) to attenuate endocytic vesicle recycling increased the effect of chemotherapy agents in a murine model, speculatively as a result of reduced EV numbers in the circulation (Chalmin *et al.* 2010). Although precise regulation of exosome release remains unclear, a number of possible therapeutic targets have been identified. Rab27b interference inhibits exosome release and can reduce tumour progression (Ostrowski *et al.* 2010, Bobrie *et al.* 2012, Peinado *et al.* 2012). Other therapeutic targets of interest include P53 and GTPases, implicated in the cytoskeleton dependent mechanism underpinning exosome exocytosis (Savina *et al.* 2005, Hsu *et al.* 2010, Zhuang *et al.* 2011). Inhibition of EV uptake into cells is also possible by blocking surface phosphatidylserine, however due to lack of specificity this mechanism it is unlikely to translate into a therapeutic intervention. Regardless of the therapeutic strategy employed, there are a large number of limitations to targeting exosome biogenesis and uptake; in particular, currently elucidated mechanisms are not tissue-specific and affect a number of complex, core functions in diseased and healthy tissue. For a future drug, this may manifest in a large number of off-target effects, greatly affecting this approach as a therapeutic strategy.

Future focus

There is great potential for uEVs as both disease biomarkers and vectors for targeted therapeutic delivery, yet we remain some way from realising this potential and improving patient outcomes. uEVs are abundant in urine, however, the contribution of EVs to normal renal physiology and their ability to modulate pathophysiological processes has yet to be proven. We still are not clear how EVs enter the urine from circulation, what the mechanisms are for targeting and how these can be effectively manipulated. The role of EVs as clinical biomarkers is perhaps closer to clinical utility. Biomarker utility needs to be confirmed in larger studies and several key challenges remain such as the development of high throughput platforms for rapid EV quantification and a consensus on normalisation across samples. These remain the same hurdles we identified previously in our 2013 review, but developments have been made through improvements to microfluidic devices and nanopore arrays (Dear *et al.* 2013). Significant challenges remain that need to be overcome if investigation and manipulation of EVs is to be translated into point-of-care diagnostics and therapeutic interventions.

Figures



Abstract figure: EV biogenesis and interaction with recipient cells. Exosomes are generally considered a homogenous population of vesicles, derived from the endosomal pathway. This process commences with the invagination of the plasma membrane and terminates when the mature MVB fuses with the limiting aspect of the plasma membrane. The contents of the MVB are liberated into the extracellular environment, releasing exosomes. Microvesicles are formed by the budding of the plasma membrane, releasing a heterogeneous population of larger vesicles. Due to their biogenesis, EVs (yellow) act as vectors for mRNA, miRNA, protein, dsDNA, mitoDNA and antigens. This parcel of biological information can be interrogated as a biomarker reservoir or propagate a signal to a recipient cell. EV interaction with a recipient cell has been described through a number of modalities, ultimately influencing a recipient cell's proteome and function. The contents can be delivered directly to the cytosol by fusion with the recipient cell membrane or through phagocytosis, macropinocytosis or clathrin-mediated endocytosis. Alternatively, the vesicles can signal by directly activating cell surface receptors via ligands or present antigen and MHC.

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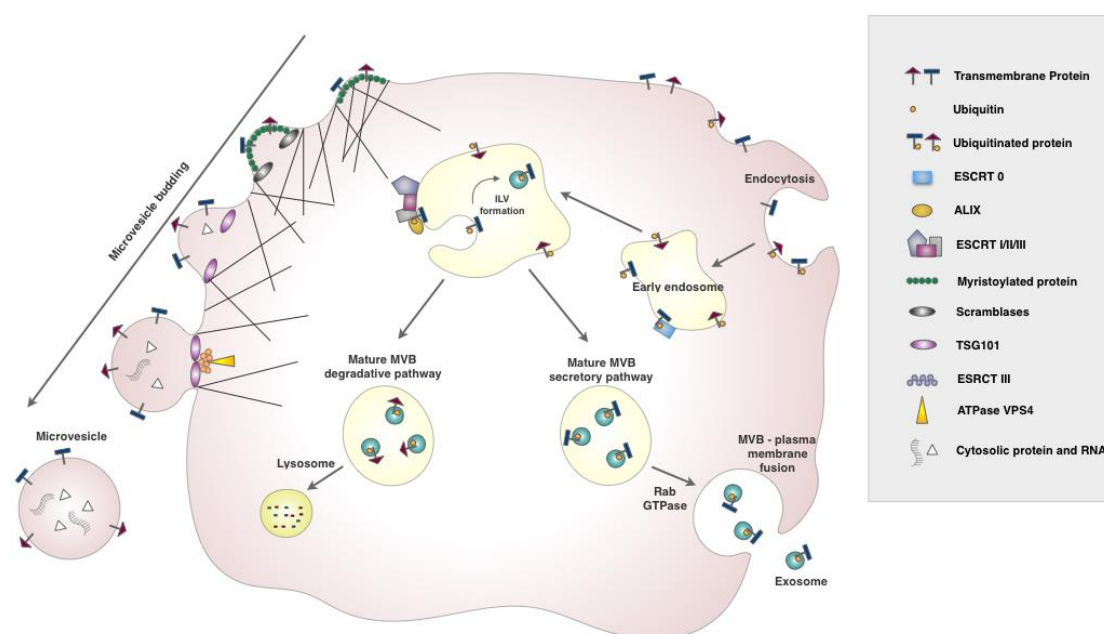


Figure 1: EV biogenesis and role of ubiquitination.

Exosome biogenesis is depicted on the right. Transmembrane proteins are internalized from the cell surface via endocytosis and their cytosolic domains ubiquitinated. ESCRT 0 recognises the ubiquitinated protein and segregates these proteins into microdomains. ESCRT I and II are subsequently recruited, by ESCRT I, and initiate the reverse budding of intraluminal vesicles (ILVs). At this stage, a same amount of cytosol, and therefore cytosolic proteins and RNA, have access to the interior of the ILV. The ILV is cleaved from the bud, following recruitment of ESCRT III, by ESCRT II and ALIX. The mature multivesicular body (MVB) formed can either follow a degradation pathway, or proceed to fusion with the plasma membrane. The pathway for each MVB is likely determined by its contents. For example, ubiquitinated LMP2A has been shown to follow the secretory pathway, while MVBs containing ubiquitinated EGFR are degraded. The degradation pathway consists of fusion of the mature MVB with a lysosome. In the secretory pathway MVBs fuse with the plasma membrane mediated by a Rab27A-dependent pathway, releasing the ILVs, now termed exosomes, into the extracellular environment. Please note, not all proteins require ubiquitination to be targeted to ILVs. Other post-translation modifications can result in recruitment to ILVs and have been reviewed in (Moreno-Gonzalo *et al.* 2014).

Microvesicle assembly is illustrated on the left and modified from (Cocucci *et al.* 2015).

Transmembrane proteins cluster in membrane lipid microdomains during nucleation at the plasma membrane. Myristoylated protein contributes to membrane curvature, lipid distribution is randomized by calcium-dependent scramblases and, concurrently, the cytoskeleton loosens. A member of the ESCRT-I complex, TSG101, recruits ESCRT III to the plasma membrane which promotes the assembly of a spiral, ultimately disassembled by ATPase VPS4. This process results in cleavage of the bud and release of a heterogeneous population of microvesicles.

Table 1: Examples of uEV biomarkers and proposed clinical usage

Disorder	Potential uEV biomarker	
	Protein	miRNA/mRNA
Acute Kidney injury		
AKI	Fetuin -A*, Activating transcription factor-3*, Na ⁺ /H ⁺ exchanger type 3*	CD2AP*†
Ischaemic reperfusion injury	Aquaporin-1*, Activating transcription factor-3*	
Glomerular injury		
Diabetic nephropathy	Dipeptidyl peptidase IV†, Podocalyxin*, Wilm's tumour-1*, Histone-lysine N-methyltransferase*, Voltage-dependent anion-selective channel protein 1*, Alpha-1-microglobulin/bikunin precursor*	miR-145*, miR-130*, miR-155*, miR-424*
Focal segmental glomerular sclerosis	Wilm's tumour 1†‡, Podocalyxin*	
Autoimmune glomerulonephritis		miR-26a†
Lupus nephritis	A disintegrin and metalloprotease 10*	miR-29c*†, miR-26a*, miR-146a*
Glomerular disease (mixed population)	A disintegrin and metalloprotease 10*	
IgA nephropathy	α1 antitrypsin*, Aminopeptidase N*, Vascularin precursor*, Ceruloplasmin*,	
Glomerular fibrosis		CD2AP*†
Other disorders		
Polycystic Kidney Disease	Polycystin-1*†, Polycystin-2*†, Polyductin*, Transmembrane protein 2*†	
Primary aldosteronism	Phosphorylated NCC*, Prostatein*	
Obstructive Nephropathy	Transforming growth factor β*	

Bartter syndrome	Na ⁺ , K ⁺ , Cl ⁻ cotransporter type 2*	
Gitelman syndrome	Phosphorylated NCC*	
Renal fibrosis		miR-29c*, miR-200*
Chronic Kidney Disease	Neutrophil gelatinase-associated lipocalin*	
Transplant	Neutrophil gelatinase-associated lipocalin†,	mRNA IL-18†
Cancer		
Prostate cancer	Integrin β1*, Integrin α3*, Prostate specific antigen*‡, Prostate specific membrane antigen*	miR-34a‡
Bladder cancer	EGF-Like Repeats And Discoidin I-Like	
	Domains 3*, Tumor-associated calcium-signal transducer 2*, Mucin 4, Epidermal Growth Factor Receptor Pathway Substrate 8-Related Protein 2*	LASS2*, GALNT1*
Renal Cell carcinoma	Matrix metalloproteinase 9*, Ceruloplasmin*, Podocalyxin*, Dickkopf related protein 4*, Carbonic Anhydrase IX*, Aquaporin-1*, Extracellular Matrix Metalloproteinase Inducer*, Neprilysin*, Dipeptidase 1*, Syntenin-1*	
Systemic illness		
Acute myocardial infarction		miR-1*, miR-208*
Parkinsons Disease	Protein deglycase DJ-1†	
Ovarian serous adenocarcinoma		miR-30a-59*
Lupus erythematosus		miR-146a*

Autoimmune encephalomyelitis		miR-155-5p*‡
Type 2 diabetes		miR-143*
NSCLC	Leucine-rich α -2-glycoprotein*	

*diagnostic †prognostic ‡response to treatment